

REMARKS

I. Preliminary Remarks

Upon entry of the instant amendment, claims 31-48, and 51-73 are pending with claims 47 and 51-66 are withdrawn for being directed to a non-elected invention. The amendment to claim 31 merely imports the definition of treatment in the specification and thus does not change the scope of the claim. Support for this amendment can be found, for example, at page 12, lines 14-16 and lines 29-31, and page 6, lines 8-9. Support for new claim 73 can be found, e.g., at page 3, lines 19-26. No new matter has been added.

II. Allowable Subject Matter

Applicants appreciate the Examiner's acknowledgement that claims 35 and 69 and allowable over the prior art and would be considered allowable if rewritten in independent form. Claim 69 was presented in an independent form in Applicants' amendment dated June 13, 2007 and is therefore directed to allowable subject matter as previously presented.

III. The rejection under 35 U.S.C. § 102(b) over Schenk should be withdrawn.

The Examiner has maintained the rejection of claims 31 and 42-45 under 35 U.S.C. § 102(b) as assertedly being anticipated by Schenk (WO 99/27944). Schenk discloses methods of "treating a disease characterized by amyloid deposition . . . [which] entail inducing an immune response against a peptide component of an amyloid deposit . . ." Page 3, lines 2-5. The induction of the immune response can either be "active by administration of an [A β] immunogen or passive by administration of an [anti-A β] antibody." Page 3, lines 6-8.

In contrast, the present invention is based on the use of A β polypeptide to promote delivery of a therapeutic non-A β polypeptide across the blood brain barrier (BBB). Claim 31 as amended recites a composition comprising an A β polypeptide linked to a therapeutic non-A β polypeptide, wherein the therapeutic non-A β polypeptide provides a clinically recognized improvement or stabilization of one or more clinical features of a CNS disorder in a statistically significant number of patients.

The only specific portion of Schenk referenced by the Examiner was pages 15-16 (see page 6 of Office Action mailed February 13, 2007). At page 16, lines 17-19 there is a discussion of immunogenic peptides, such as A β , fused to bacterial or viral proteins (such as surface or transmembrane proteins) so that the immunogenic peptide is displayed.

Applicants disagree with the Examiner's position that page 16 further describes "fusion peptides including antibodies" (page 6 of February 13, 2007 Office Action). The discussion relating to fusions (page 16, lines 11-22) is *separate from* and unrelated to the discussion of antibodies. The reference to antibodies at page 16, line 28-31 is part of a discussion of a category of therapeutic agents that are mimetics of A β ("anti-Id[iotypic] antibodies [that] mimic the antigen").

Schenk also discloses compositions comprising A β or an active fragment linked to a conjugate molecule (e.g., antibody, cholera toxin or attenuated diphtheria toxin) that either "promotes delivery of A β to the bloodstream of a patient and/or promotes an immune response against A β ."

Page 4, lines 35-37.

Anticipation requires that the cited art disclose each and every element of the claims, which is not the case here. *Schenk does not anticipate the claimed invention because Schenk fails to disclose an A β polypeptide linked to a non-A β polypeptide that is a therapeutic for a CNS disorder, as recited in claim 31.* According to Schenk, the fusion or conjugate polypeptide (i.e., the non-A β polypeptide) is (1) a bacterial or viral protein that displays the A β polypeptide on the surface of the bacteria or virus, (2) a molecule that promotes delivery of A β to the bloodstream, or (3) a molecule that promotes an immune response against A β .

The Examiner has not pointed to any teaching in Schenk that the polypeptide fused or conjugated to A β (i.e., the non-A β polypeptide) provides a clinically recognized improvement or stabilization of one or more clinical features of a CNS disorder in a statistically significant number of patients, as recited in claim 31. Moreover, the Examiner has failed to provide any evidence to establish that any of these molecules is inherently a therapeutic for a CNS disorder according to claim 31.

As explained in MPEP §2112, "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is *necessarily present* in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'" *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted, emphasis added). "In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic *necessarily* flows from the teachings of the

applied prior art.” Ex parte Levy, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis in original).

To the extent that the Examiner’s position appears to be that the recitation of non-A β polypeptides “for treatment of CNS disorders” is so broad as to encompass any polypeptide, the Examiner has not properly considered the definition of treatment in the specification. In order to clarify claim 31 and address the Examiner’s concerns, Applicants have amended the claim to incorporate the definition already set forth in the specification, i.e., that the treatment result in a clinically recognized improvement or stabilization of one or more clinical features of a CNS disorder in a statistically significant number of patients.

Thus, Applicants respectfully submit that there is no proper case of anticipation with respect to claim 31 because there is no evidence that the conjugate or fusion polypeptides described in Schenk are for treating CNS disorder as that term is defined in the specification and in the claim. In any event, a *prima facie* case of anticipation can be rebutted by evidence showing that the prior art products do not necessarily possess the characteristics of the claimed product. Such rebuttal evidence is provided by the disclosure of Schenk itself, which teaches that the conjugate or fusion polypeptides are for entirely different purposes than treating CNS disorders (i.e., for displaying the A β polypeptide, promoting delivery to the bloodstream, or promoting an immune response).

Applicants note that the Examiner has not pointed to a specific disclosure in Schenk of the modifications at particular positions claimed in claim 45, and therefore there is not a proper case of anticipation with respect to claim 45.

Applicants note that new claim 73 is parallel to claim 31 but additionally recites a number of specific CNS disorders, in addition to the limitation that the non-A β polypeptide provides a clinically recognized improvement or stabilization of one or more clinical features of a CNS disorder in a statistically significant number of patients. There is no evidence that any of the conjugate or fusion polypeptides disclosed in Schenk is a therapeutic for the listed CNS disorders according to claim 73.

In view of the foregoing, Applicants respectfully request that the rejection of claims 31 and 42-45 under 35 U.S.C. §102(b) be withdrawn.

IV. The rejection under 35 U.S.C. § 102(b) over Wu should be withdrawn.

The Examiner rejected claims 31, 33, 34, 42, 43, 48, 67-69 and 72 under 35 U.S.C. § 102(b) as assertedly being anticipated by Wu et al. (J. Clin. Invest., 100:1804-1812, 1997).

Wu discloses a composition comprising a drug delivery system composed of a conjugate of $^{125}\text{I}-\text{A}\beta^{1-40}$, streptavidin, biotin and the 83-14 monoclonal antibody to the human insulin receptor, wherein the 83-14 monoclonal antibody is intended to deliver the composition across the blood brain barrier (BBB). See page 1805, 1st column, lines 13-14. The present invention, however, reports that the $\text{A}\beta$ polypeptide itself is the transport vehicle for the non- $\text{A}\beta$ polypeptide. Wu further discloses that its delivery system is used for imaging the distribution of the $\text{A}\beta$ peptide radiopharmaceutical in the brain. In contrast, the present invention is drawn to a therapeutic composition for the treatment of a human patient that has been diagnosed with a CNS disorder. Wu provides no disclosure for the treatment of any subject that has been diagnosed with a CNS disorder.

Anticipation requires that the cited art disclose each and every element of the claims, which is not the case here. There is no teaching in Wu that any of streptavidin, biotin or the 83-14 monoclonal antibody to human insulin receptor provides a clinically recognized improvement or stabilization of one or more clinical features of a CNS disorder in a statistically significant number of patients. Moreover, the Examiner has failed to provide any evidence to establish that the 83-14 monoclonal antibody is inherently a therapeutic for a CNS disorder according to claim 31. Thus, Applicants respectfully submit that there is no proper case of anticipation with respect to claim 31. Moreover, a *prima facie* case of anticipation would be rebutted in view of the disclosure of Wu itself, which teaches that the 83-14 monoclonal antibody is *not* for treating a CNS disorder but rather is merely a delivery vehicle for the radiopharmaceutical. Consequently, Wu does not anticipate any of claims 31-34, 42 and 43.

With respect to claims 42-43 and 67-68, Wu does not provide the basis for an anticipation rejection for these claims because Wu does not teach that the $\text{A}\beta$ polypeptide is *covalently linked* to a non- $\text{A}\beta$ polypeptide. In contrast, the $\text{A}\beta^{1-40}$ is non-covalently bound to the 83-14 monoclonal antibody through a non-covalent association between streptavidin and biotin. It is well known in the art that the interaction between streptavidin and biotin is a *non-covalent* linkage. See, for example, the abstract of Weber et al., Science, 243:85-88 (1989), set forth as Appendix A. Accordingly, because Wu does not disclose an $\text{A}\beta$ polypeptide that is *covalently linked* to a non- $\text{A}\beta$ polypeptide, it does not disclose each and every element of claim 42 or 67 and therefore cannot destroy the novelty of claim 42 or 67 or those claims dependent thereon.

Claim 69 was indicated elsewhere to be directed to allowable subject matter.

Turning now to the rejection of claim 72, the Examiner has not pointed to any specific teaching in Wu which discloses that the composition disclosed therein “exhibits a permeability coefficient x surface area (PS) product of 2.3×10^{-6} ml/g/sec or greater, wherein the PS product is determined after correction for the residual plasma volume (Vp) occupied by the protein in blood vessels in different brain regions following an intravenous bolus injection” as recited in claim 72. Applicants’ review of Wu did not find such a teaching. Accordingly, Wu does not anticipate claim 72.

Wu also does not anticipate new claim 73, as there is no evidence that the 83-14 monoclonal antibody provides a clinically recognized improvement or stabilization of one or more clinical features, in a statistically significant number of patients, for any of the CNS disorders listed in claim 73.

In view of the foregoing, Applicants respectfully request that the rejection of claims 31, 33, 34, 42, 43, 48, 67-69 and 72 under 35 U.S.C. §102(b) be withdrawn.

V. The rejection under 35 U.S.C. § 103(a) should be withdrawn.

The Examiner rejected claims 36-41, 46 and 70-71 as assertedly being obvious over Wu in view of Schenk. Applicants disagree.

It appears from the Office Action that the Examiner rejected claims 36-41 as assertedly being obvious over Wu in view of knowledge in the art, while claims 46 and 70-71 were rejected as assertedly being obvious over Wu in view of Schenk.

In the Office Action, the Examiner contended that the art of antibody modification is well developed and that it would have been obvious at the time of the invention to substitute the monoclonal antibody of Wu for an antibody fragment or chimeric antibody to arrive at the claimed invention. The Examiner’s assertion is irrelevant because the combination of the knowledge of the art with respect antibody modification and the disclosure of Wu would not result in the claimed invention because, as discussed above in Section IV, Wu does not disclose or suggest a therapeutic composition according to claim 31. Accordingly, dependent claims 36-41 are nonobvious over Wu.

Moreover, one of ordinary skill in the art would not have been motivated to arrive at the claimed invention based on Wu because doing so would go *against the express teachings* of Wu.

Wu does not disclose or suggest that the A β polypeptide itself is capable of enhancing transport of a non-A β polypeptide across the BBB. Rather, Wu teaches that "...no measurable uptake of ^{125}I -A β ¹⁻⁴⁰ was observed in the absence of a BBB drug delivery system" (Wu, abstract). Therefore, upon review of Wu, one of skill in the art would be led to avoid using A β as a transport vehicle to cross the BBB and correspondingly would not be motivated to conjugate A β to a therapeutic agent for CNS disorders. Furthermore, because Wu reports that unconjugated A β undergoes negligible transport across the BBB, one of skill in the art would find no reasonable expectation of success in Wu to arrive at the claimed invention.

Schenk fails to provide the disclosure lacking from Wu. Notably, Schenk does not disclose or suggest that the A β polypeptide itself is the delivery vehicle and is silent with respect to the delivery across the BBB. Moreover, Schenk does not specifically disclose or suggest that a patient having received its conjugate composition would exhibit a clinically recognized improvement or stabilization of one or more clinical features of a CNS disorder in a statistically significant number of patients.

The Examiner asserts that it would have been obvious for a person of ordinary skill in the art to modify the composition of Wu by changing the structure of A β as indicated by Schenk and reasonable expect the resulting product to be the same. Applicants disagree. While the Examiner has indicated that Schenk generically discloses amino acid modifications of the A β polypeptide, the Examiner has failed to point out the specific teaching in Schenk of the specific amino acid modifications recited in claims 46, 70 and 71.

Therefore, in view of Wu's teaching away from the claimed invention, Schenk's failure to specifically point out the specific amino acid modifications recited in the claims and the lack of reasonable expectation of success, Applicants respectfully submit that the claims are nonobvious over Wu, and the combined disclosure of Wu and Schenk. Reconsideration and withdrawal of the rejection is respectfully requested.

VI. Conclusion

It is believed that the foregoing responds to all of the Examiner's concerns. If the Examiner believes that a telephone conversation would expedite allowance of the claims, she is invited to contact the undersigned agent or Li-Hsien Rin-Laures, Attorney for Applicants, at the number below.

Application No. 10/796,522
Amendment dated October 30, 2007
Reply to Office Action of August 28, 2007

Docket No.: 01017/30016A

The Director is hereby authorized to charge any additional fees associated with the filing of this paper to Deposit Account No. 13-2855, under order no. 01017/30016A.

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Respectfully submitted,

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APPENDIX A

Egg albumin (Sigma) and Pertussis (Connaught Laboratories) were administered according to protocol described in M. H. Perdue, M. Chung, D. G. Gall, *Gastroenterology* **86**, 391 (1984).

- K. J. King *et al.*, *Eur. J. Immunol.* **16**, 151 (1986); S. J. King and H. R. P. Miller *Immunology* **51**, 653 (1984).
- K. J. Bloch *et al.*, *Gastroenterology* **77**, 1039 (1979); E. Jarrett and H. Bazin, *Nature* **251**, 613 (1974); *Clin. Exp. Immunol.* **30**, 330 (1977).
- Animals were removed from the colony room and placed in plastic cages inside concrete-enclosed (soundproof) cabinets. A light flashed at an alteration rate of 300 ms, and background noise was provided by ventilation fans. The AV CS was based on that used by G. MacQueen and S. Siegel (*Behav. Neurosci.*, in press).
- An enzyme-linked immunosorbent assay (ELISA) for detecting RMCP II was modified from Miller *et al.* (7). Rats were anesthetized with ether, and blood was obtained from the retro-orbital plexus. Sera were collected and stored at -20°C. The wells of a tissue culture microtiter plate (Nuncolon Delta) were coated with 0.5 mg of RMCP II per milliliter of a 0.2M carbonate buffer, pH 9.6. Samples and standards were diluted in PBS containing 0.3% w/v bovine serum albumin, 0.02% v/v polyoxyethylene-sorbitan monolaurate (Tween 20), and 0.02% w/v sodium azide and incubated for 16 to 24 hours with a diluted specific rabbit antiserum to RMCP II (anti-RMCP II) from which all activity against RMCP I had been removed by immunoadsorption. After extensive washing of the plates, 100 µl of samples and standards were placed in duplicate wells and incubated for 16 to 24 hours. Plates were again washed several times and rabbit antibody bound to the plate was detected with an alkaline phosphatase-conjugated goat to rabbit antibody (ICN) and a sodium-p-nitrophenyl phosphate substrate (Sigma). Results were calculated on the basis of a standard curve constructed with the use of known concentrations of purified RMCP II. Antibodies to RMCP I were raised in rabbits with purified RMCP II. Antisera were absorbed twice with RMCP I covalently attached to Sepharose 4B. The final preparation showed strong binding to RMCP II on immunoblot analysis, with a weak cross-reaction to RMCP I, and no detectable binding to cathepsin G. The specificity of this assay was confirmed with homogenates of tongue tissue known to contain high levels of RMCP I, but no RMCP II. Although there was some binding to RMCP I on the immunoblot, no binding was detected in the liquid phase.
- Morphological studies have shown the presence of mast cells in peripheral nerves [Y. Olsson, *Acta Neurol. Scand.* **47**, 357 (1971)] and autonomic ganglia [G. Gabella, *Structure of the Autonomic Nervous System* (Chapman and Hall, London, 1976)], a consistent ultrastructural relationship between mucosal mast cells and nerves in normal and nematode-infected rat lamina propria was detected [R. H. Stead, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2975 (1987)].
- Substance P causes release of histamine from mast cells in vitro [F. Shanahan *et al.*, *J. Immunol.* **135**, 1331 (1985); O. Hagermark, T. Hockfelt, B. Persson, *J. Invest. Dermatol.* **71**, 233 (1978)], and mast cells and substance P containing nerves may be involved in the vasodilatory response to noxious stimuli [J. C. Foreman and C. C. Jordan, *J. Physiol.* **238**, 58 (1982)].
- Evidence has also supported a role for functional nerve-mast cell interactions. A. R. Leff *et al.* [*J. Physiol.* **136**, 1066 (1986)] demonstrated that vagal stimulation causes enhanced histamine release from mast cells after antigen challenge, and a decrease in mast cell granularity has been shown after electrical field stimulation [T. Bani-Sacchi *et al.*, *J. Physiol.* **371**, 29 (1986)]. Studies of hypersensitivity reactions in the gut and lung indicate a neural component in the changes in epithelial ion transport induced by antigen [Y. Harari *et al.*, *J. Immunol.* **138**, 1250 (1987); see M. H. Perdue in (10)].
- D. Befus, F. Pearce, J. Bienenstock, in *Food Allergy and Intolerance*, J. Brostoff and S. J. Challacombe, Eds. (Baillière-Tindall, London, 1986), pp. 88-98;
- E. G. Seidman, D. G. Hanson, W. A. Walker, *Gastroenterology* **90**, 120 (1986); J. B. Kirsner and R. G. Shorter, *N. Engl. J. Med.* **306**, 837 (1982).
- J. C. Foreman, *Allergy* **42**, 1 (1987); D. M. Barnes, *Science* **232**, 160 (1986); P. J. Barnes, *Lancet* **i** 242 (1986).
- Supported by grants from the Medical Research Council of Canada, the Natural Sciences and Engineering Research Council of Canada, and the Foundation for Ileitis and Colitis. We thank L. Neilson for technical assistance and R. Woodbury for the RCMP II and the anti-RCMP II.

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Structural Origins of High-Affinity Biotin Binding to Streptavidin

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The high affinity of the noncovalent interaction between biotin and streptavidin forms the basis for many diagnostic assays that require the formation of an irreversible and specific linkage between biological macromolecules. Comparison of the refined crystal structures of apo and a streptavidin:biotin complex shows that the high affinity results from several factors. These factors include the formation of multiple hydrogen bonds and van der Waals interactions between biotin and the protein, together with the ordering of surface polypeptide loops that bury the biotin in the protein interior. Structural alterations at the biotin binding site produce quaternary changes in the streptavidin tetramer. These changes apparently propagate through cooperative deformations in the twisted β sheets that link tetramer subunits.

STREPTAVIDIN IS A TETRAMERIC PROTEIN (molecular weight = $4 \times 15,000$) isolated from the actinobacterium *Streptomyces avidinii* (1). Streptavidin, and the homologous protein avidin, are remarkable for their ability to bind up to four molecules of *d*-biotin with unusually high affinity [dissociation constant $K_d = 10^{-15} M$ (1, 2)]. Although these proteins may function as antibiotics that deplete the environment of the essential vitamin biotin, they have been studied primarily as paradigms for understanding high-affinity protein-ligand interactions (2). At the same time, the ability of streptavidin and avidin to bind derivatized forms of biotin has led to their widespread use in diagnostic assays that require formation of an essentially irreversible and specific linkage between biological macromolecules (3). We undertook the structure determination of streptavidin, with and without bound biotin, to uncover the origins of high affinity of the protein for biotin.

Streptavidin was obtained from several commercial sources and produced different crystal forms during the course of the study. The most consistent results were obtained with a fragment of the native 159-residue streptavidin chain, incorporating residues 13 through 133. Numerous studies indicate that this truncated form of the molecule

binds biotin with an affinity that is the same or similar to alternative longer versions of the protein. Moreover, in some cases it appeared that preparations identified as full-length material crystallized isomorphously with the truncated fragment, suggesting that the molecular termini may be relatively flexible or disordered. Crystallization conditions for apostreptavidin and its biotin complex were found by robotic grid search methods (4). Both formed crystals from a polyethylene glycol-LiCl mixture, although the streptavidin:biotin complex crystallizes at pH 7.8 [space group $I4_122$, $a = b = 99.4 \text{ \AA}$, $c = 125.8 \text{ \AA}$ (5)], whereas apostreptavidin crystallizes at pH 2.4 [space group $I4_122$, $a = b = 58.3 \text{ \AA}$, $c = 172.5 \text{ \AA}$]. Unit cell parameters of truncated apostreptavidin are similar to those reported by Pahler *et al.* (6), although the crystals studied here grow at lower pH, and diffract to higher resolution ($d_{\min} = 1.7 \text{ \AA}$).

The structure of apostreptavidin was determined by multiple isomorphous replacement techniques. X-ray diffraction data for parent crystals and several isomorphous replacement derivatives were collected using a multiwire area detector and processed with the Xengen data-reduction package (7). Successful derivatives included $K_2Pt(SCN)_6$, which was prepared by soaking crystals in the heavy metal solution, and an iodine derivative prepared by crystallizing protein after reaction in solution (8). Substitution sites were located by an automated search procedure (9) performed on the heavy atom difference Patterson maps. Phases were ob-

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tained after refinement of heavy atom positions by the origin-removed different Patterson method (10) and used to compute a 2.6 Å resolution electron density map (11).

The electron density map was interpreted with the graphics program FRODO (12). Identification of several Trp and other large residues allowed an initial α -carbon backbone trace and sequence assignment to be made for $\sim 70\%$ of the structure, organized primarily as antiparallel β sheet. A partial atomic model was constructed from the α -carbon trace with the use of fragment superposition (13) from a database of refined protein structures (14). Complete backbone fragments that best fit the α -carbon trace were incorporated into the model. Side chains were positioned by displaying possible rotamers for each amino acid from a library (15) and by including coordinates for the rotamer that best fit the electron density. Phases computed from the partial model were combined with the multiple isomorphous replacement phases and used to generate an improved electron density map whose interpretation defined the remainder of the molecular structure. The structure was refined with the restrained least-squares method of Hendrickson and Konnert (16) and manual rebuildings in electron density maps. Although the refinement proceeded smoothly, two surface loops lacked defined density and appeared disordered in the final structure. The crystallographic *R*-factor for apostreptavidin, including residues 13 to 46, 49 to 63, and 69 to 133, as well as 33 water molecules, is 0.21 for 11,120 reflections [$F_{\text{observed}} > \sigma(F_{\text{observed}})$] between 5.0 and 1.8 Å resolution.

The structure of the streptavidin:biotin complex was solved with the use of symmetry-constrained searches of the complex unit cell with the apostreptavidin tetramer (17). Apostreptavidin crystallizes with a monomer in the asymmetric unit, so that subunits of the tetramer are related by crystallographic dyad axes. Since tetramers in the nonisomorphous crystals of the streptavidin:biotin complex could also pack with subunits related by crystal symmetry, we searched that cell using the apostreptavidin tetramer as the probe molecule (18). A maximum correlation coefficient of 0.56 was obtained for 1363 intensities between 4 and 5 Å resolution when the streptavidin tetramer was positioned at the origin of the streptavidin:biotin complex unit cell with all three of its dyad axes coincident with the crystallographic dyad axes. This result shows that both apo and liganded forms of streptavidin are tetramers with subunits related by 222 point group symmetry. The initial crystallographic *R*-factor for apostreptavidin positioned in the biotin:streptavidin unit cell

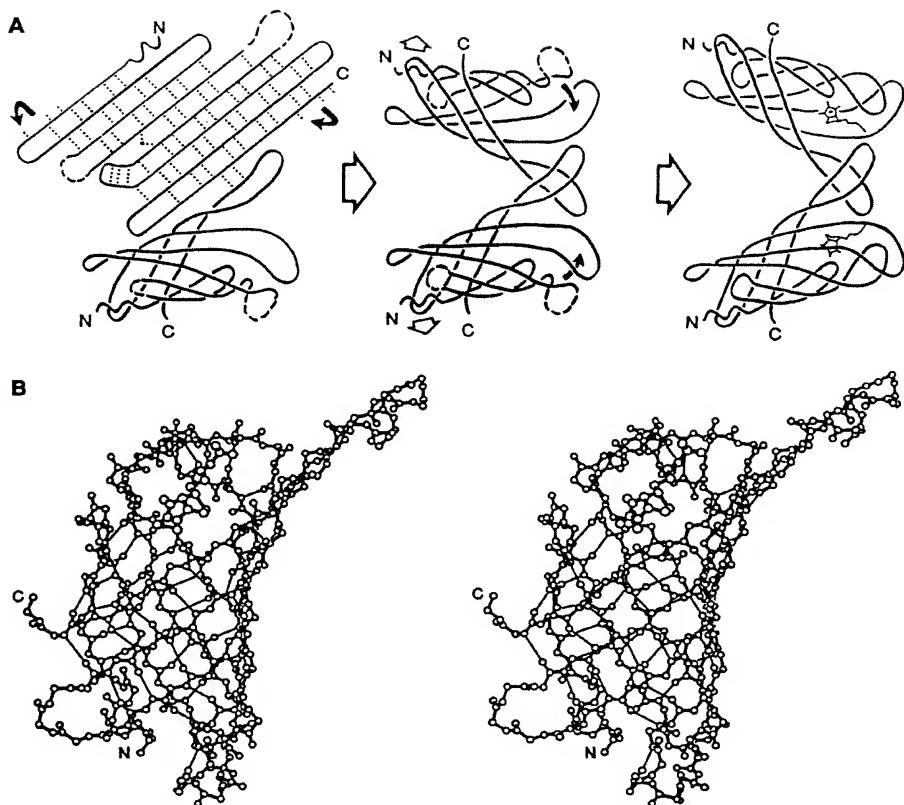


Fig. 1. Streptavidin structure. (A) Cartoon sequentially showing the β sheet folding pan of the hydrogen-bonded dimer, the apostreptavidin structure, and changes upon biotin binding. These changes include ordering of two loops (shown dashed) incorporating residues 45 to 50 and 63 to 69. (B) Stereoview of a streptavidin subunit with biotin bound, showing β barrel hydrogen bonds as thin lines. Residues 13 through 133 form an eight-stranded antiparallel β sheet wrapped as a slightly flattened barrel.

was 0.41 for data from 5.0 to 2.6 Å resolution.

The structure of the streptavidin:biotin complex was refined by a combination of conventional restrained least-squares methods and crystallographically constrained molecular dynamics. The molecular dynamics refinement protocol essentially followed previous work (19), but was implemented in our laboratory by combining features of AMBER (20), PROLSQ (16), and PROFFT (21). The crystallographic *R*-factor of the streptavidin:biotin complex, including all residues between sequence positions 13 and 133, biotin, and nine water molecules, is 0.22 for 7379 reflections with $F_{\text{observed}} > \sigma(F_{\text{observed}})$ between 5.0 and 2.6 Å resolution [coordinates will be deposited in the Brookhaven Protein Data Bank (14)].

Streptavidin subunits are organized as eight-stranded, sequentially connected, antiparallel β sheets. The sheets are formed of coiled polypeptide chains with a staggered pattern of adjacent strand hydrogen-bond registration (22). This arrangement pro-

duces a cyclically hydrogen-bonded barrel with several extended hairpin loops, including one near the carboxyl terminus whose edge is free to form a more extended β sheet (Fig. 1). Pairs of streptavidin barrels hydrogen bond together at this free edge to form symmetric dimers that resemble basketball nets connected by their rims at a 45° angle. The naturally occurring streptavidin tetramer is formed by interdigitating a pair of dimers, with their dyad axes coincident, to produce a particle with 222 point group symmetry. The tetramer is stabilized by extensive van der Waals interactions between the subunit barrel surfaces, which have complementary curvatures (Fig. 2).

Biotin binds in pockets at the ends of each of the streptavidin β barrels (Fig. 1). The residues lining the pockets are primarily aromatic or polar amino acids or both. These groups are solvent exposed in apostreptavidin so that several water molecules occupy the biotin binding site. Biotin binding involves displacement of bound water, formation of multiple interactions between biotin heteroatoms and the binding site

residues, and burial of the biotin through ordering of a surface loop (residues 45 to 50) that is disordered in streptavidin. Polar interactions made between biotin heteroat-

oms and the protein include (i) an extensive pattern of hydrogen bonds with the biotin ureido group, where no less than five protein residues form associations; (ii) a possi-

ble interaction between biotin sulfur and the hydroxyl group of Thr⁹⁰; and (iii) hydrogen-bonded interactions with the valeryl carboxyl group that includes a hydrogen bond from the backbone NH of Asn⁴⁹, which becomes ordered on biotin binding (Fig. 3). Several other residues lining the binding site are immobilized by hydrogen bonds, which are formed in many cases with the same residues that hydrogen bond to biotin. These include Trp residues 79, 92, and 108 that pack around the biotin tetrahydrothiophene ring, and which, together with Trp¹²⁰ from the cyad-related subunit, form a hydrophobic biotin binding site. As a result of this extensive pattern of interactions, resulting in part from the ordering of loop 45 to 50, bound biotin is essentially buried in the complex with only the valeryl carboxyl oxygens partially accessible to solvent.

Experimental studies of the binding of biotin analogs to avidin, a tetrameric protein from avian egg white that shares 38% sequence identity (23) with the crystallographically defined streptavidin, suggest that interactions made with the ureido ring system predominate in stabilizing the biotin-protein complex (2). An unusual aspect of the interaction involves participation of the bio-

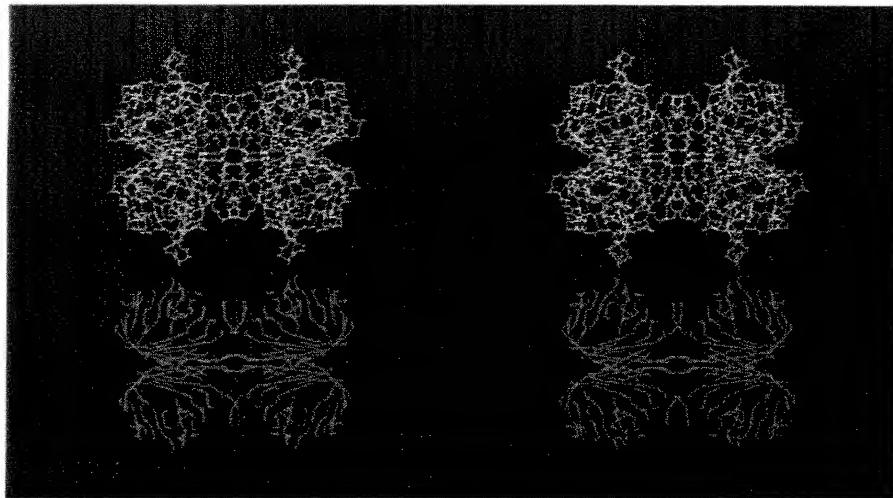


Fig. 2. Stereoviews of the streptavidin tetramer. (Top) Tetramer with bound biotins (backbone atoms), viewed along dyad symmetry axis relating hydrogen-bonded subunits (hydrogen bonds in red). (Bottom) Hydrogen-bond circuit representation of the tetramer, as defined by sheet hydrogen bonds and backbone amide group atoms. This representation emphasizes the continuity of the interactions that distribute forces throughout the barrels and across the subunit dimer axes.

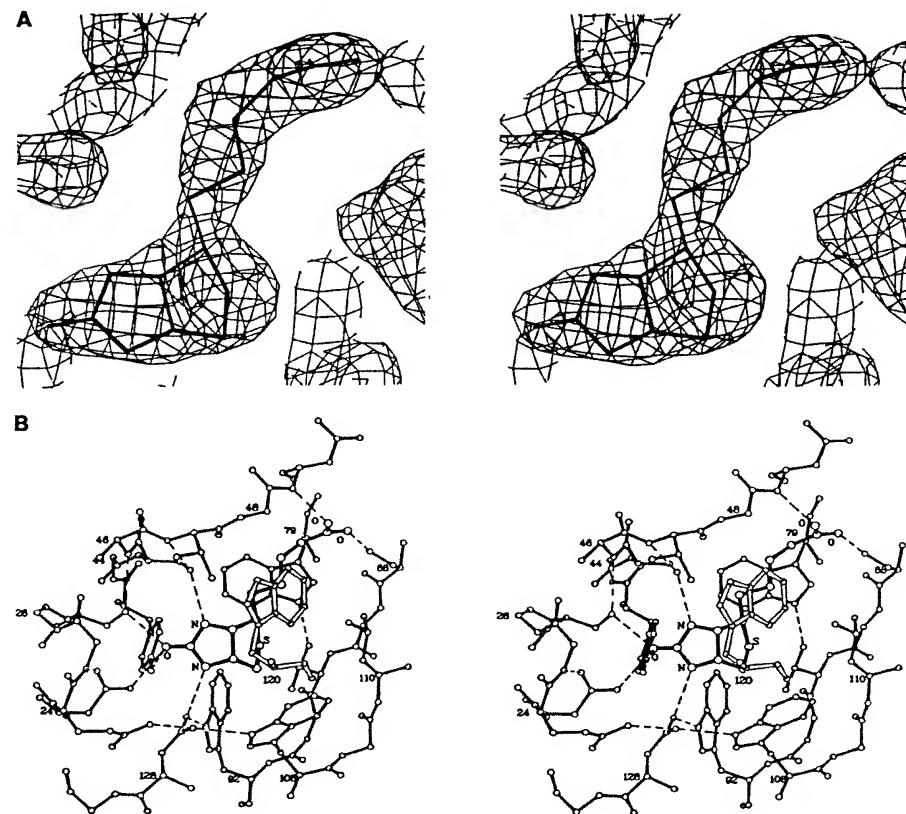


Fig. 3. Biotin binding site. (A) Biotin electron density from final $(2F_0 - F_c)\alpha_{\text{calc}}$ 2.6 Å electron density map. (B) Biotin interactions. One indole ring (open bonds) is contributed by Trp¹²⁰ from the symmetry-related, hydrogen-bonded dimer. Residues shown include: Asn²³, Gln, Leu, Gly, Ser²⁷, Tyr⁴³, Glu, Ser, Ala, Val, Gly, Asn⁴⁹, Trp⁷⁹, Ser⁸⁸, Ala, Thr, Thr, Trp⁹², Trp¹⁰⁸, Leu, Leu¹¹⁰, Asp¹²⁸, and Thr¹²⁹. Some side chains are omitted for clarity. Dashed lines show the hydrogen-bonding pattern for residues that interact with biotin heteroatoms. The valeryl oxygens of biotin are hydrogen-bonded to the backbone NH of Asn⁴⁹ and Oyl of Ser⁸⁸, respectively. The Thr⁹⁰ side chain oxygen is near the biotin sulfur and Trp⁷⁹ Nε1. Ser⁴³ Oyl interacts with one biotin ureido NH (upper) and the backbone NH of Val⁴⁷. The other biotin ureido NH (lower) is hydrogen-bonded to a carboxyl oxygen of Asp¹²⁸. The Asp¹²⁸ side chain oxygens form additional interactions with Trp⁹² Nε1, Trp¹⁰⁸ Nε1, and Gln²⁴ Nε1. Three side chain atoms, Tyr⁴³ OH, Asn²³ NH, and Ser²⁷ OH, are situated to hydrogen bond with the biotin ureido oxygen. The OH of Ser²⁷ is also hydrogen-bonded to Ala⁴⁴ NH, and Asn²³ Oyl interacts with Leu²⁵ NH. Note that residues forming hydrogen bonds with biotin ureido oxygen are themselves stabilized by orienting hydrogen bonds to backbone NH groups.

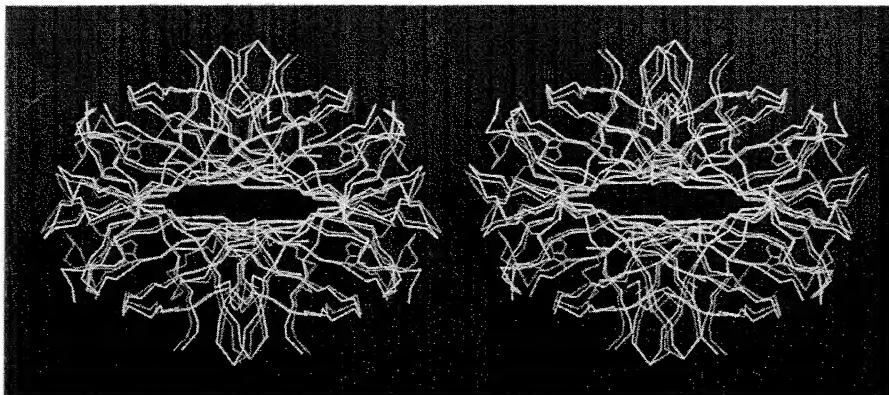


Fig. 4. Streptavidin quaternary structural changes. Yellow lines show α -carbon backbone trace of apo-streptavidin and blue lines show streptavidin with biotin bound. Pairs of hydrogen-bonded dimers have been separated by 15 Å along the horizontal tetramer dyad axis for clarity. Changes in the hydrogen-bonded dimer geometry produce relative rotations of the dimers about the horizontal dyad. Tetramer subunits of both the apo-streptavidin and streptavidin:biotin complex crystals are related by crystal-symmetry operations. The root-mean-square (rms) displacement for 112 common subunit $\text{C}\alpha$ atoms, relative to the origin defined by the intersection of the tetramer 222 symmetry axes is 2.0 Å; the rms fit for the 67 $\text{C}\alpha$ atoms in the β barrel is 0.7 Å in this frame. Superpositioning individual subunits gives an rms of 1.9 Å for all 112 $\text{C}\alpha$ atoms common to the apo and liganded proteins and 0.3 Å for the 67 β barrel $\text{C}\alpha$ atoms.

tin ureido group in an extended hydrogen-bond network anchored by the buried carboxyl group of Asp¹²⁸ that hydrogen bonds one ureido NH. The latter hydrogen bond could stabilize resonance forms that localize positive charge on biotin nitrogens and negative charge at the biotin ureido oxygen. Indeed, the ureido oxygen forms three hydrogen bonds, arranged with tetrahedral geometry, suggesting that the groups involved stabilize an sp^3 oxyanion (Fig. 3B). Comparison of streptavidin and avian avidin show that all of the groups that directly bind biotin are conserved with the exception of Ser⁴⁵, which is replaced by Thr with similar functionality, and Asp¹²⁸, which, surprisingly, is substituted by Asn (23). These changes may reflect some differences in the way biotin is stabilized in streptavidin and avidin. However, the analog of the residue that hydrogen bonds to Asp¹²⁸ in streptavidin, Gln²⁴, is substituted by Asp in avidin, so that slightly different but similar polarization networks could be functional in both proteins. Although biotin makes additional hydrophobic and hydrogen-binding interactions that assist binding, the hydrogen-bonded interactions with the valeryl group appear to play a lesser role. This group is partially accessible in the complex and provides the covalent attachment sites for linking biotin with other biomolecules (3).

Apo and liganded streptavidin differ in quaternary structure. Although the observed changes could in part reflect differences in crystal pH or lattice interactions and there is currently no evidence for subunit cooperativity, the pattern of quaternary changes

nevertheless suggests a consistent mechanism of subunit communication. Subunit differences between apo and liganded streptavidin include the formation of extensive biotin:protein interactions, concomitant ordering of two surface loops, and formation of a salt link between Glu⁵¹ and Arg⁸⁴ from adjacent loops. Collectively, these interactions cause the subunit barrels to flatten slightly and become more tightly wrapped. Because the subunit barrels are part of a more extended β sheet that forms the hydrogen-bonded dimer (Figs. 1 and 2), and the barrel exteriors pack at the dimer-dimer interface (Figs. 2 and 4), changes in barrel curvature effect both hydrogen-bonded dimer geometry and dimer-dimer packing. The net result of the change in subunit barrel curvature is to alter the twist of β sheet that connects dimer subunits, which produces a slight increase in the angle between the barrel domains. The tetramer adjusts to the changes in dimer sheet twist and preserves the complementary sheet packing by a 5.4° rotation of the dimer subunits around the corresponding tetramer dyad axis (Fig. 4).

The unusually high affinity of streptavidin for biotin reflects participation of a number of factors, the analogs of which have been previously encountered individually in other protein-ligand interactions. These factors include oriented dipole arrays to stabilize bound oxyanions [for example, the oxyanion hole in serine proteases (24)], hydrogen-bond dipole networks to alter charge distribution on bound ligands [such as the serine protease charge relay system (24)], and dis-

order-order transitions to sequester bound ligands from the solvent environment [as in triose phosphate isomerase (25)]. In streptavidin, these factors, together with quaternary changes in structure, combine to produce both strong binding and a high activation energy for dissociation that characterize the near irreversibility of the biotin:streptavidin interaction.

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11. The agreement between multiple observed diffraction intensities, $R_m = \{\text{rms} [(I_j - \langle I \rangle)/\sigma_j]\} / (\text{rms}, \text{root-mean-square})$, for 84,062 observations of 9,225 unique reflections to 2.6 Å resolution was 0.054 for the streptavidin:biotin complex, and the R_m for apo-streptavidin native crystals was 0.052 for 89,393 observations of 13,496 observations to 1.7 Å resolution. The fractional difference in diffraction amplitudes to 2.2 Å resolution between native and derivative crystals of apo-streptavidin was 0.14 for iodinated apo-streptavidin and 0.19 for the crystal soaked in $\text{K}_2\text{Pt}(\text{SCN})_6$. In the apo-streptavidin structure determination by multiple isomorphous replacement techniques (10), the mean figure of merit for 4640 reflections to 2.6 Å resolution was 0.62, and the ratio of the mean heavy atom scattering factor to the mean lack of closure (f_H/E) was 1.4 for acentric reflections of the platinum derivative and 0.8 for those of the iodinated apo-streptavidin.
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